

THE COMPLETE GENOME AND PROTEIN SEQUENCE OF THE
HYPERTHERMOPHILE METHANOPYRUS KANDLERI AV19 AND MONOPHYLY
OF ARCHAEL METHANOGENS AND METHODS OF USE THEREOF

CONTRACTUAL ORIGIN OF INVENTION

5 This work was supported in part by DOE and NIH grants (DE-FG02-98ER82577, 00ER83009, R44GM55485, R43HG02186) to S.A.K and A.I.S.

CROSS-REFERENCE TO OTHER APPLICATIONS

This patent claims priority to U.S. Provisional Patent applications
60/361,742 filed March 4, 2002 and 60/410,974 entitled "Helix-hairpin-helix motifs to
10 manipulate properties of DNA processing enzymes," filed September 16, 2002, both
of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention:

This invention relates to novel methods of sequencing directly from
15 genomic DNA. In particular, the genomic DNA of the bacterial species *Methanopyrus
kandleri* AV19 was unlinked with ThermoFidase version of *M. kandleri*
topoisomerase V and its entire nucleotide sequence was determined by directed
cycle sequencing using 2'-modified oligonucleotides (Fimers). The resulting genomic
sequences, protein sequences from *M. kandleri* and there uses in research and
20 diagnostics fields are herein disclosed.

2. Description of the State of Art:

Methanopyrus kandleri was isolated from the sea floor at the base of a
2,000 meter-deep "black smoker" chimney in the Gulf of California (Huber, R., *et al.*,
Nature, **342**:833-6 (1989)). The organism is a rod-shaped, Gram-positive
25 methanogen that grows chemolithoautotrophically at 80 to 110°C in the H₂-CO₂
atmosphere (Kurr, M., *et al.*, *Arch Microbiol*, **156**:239-47(1991)). The discovery of
Methanopyrus showed that biogenic methanogenesis was possible above 100°C and
could account for isotope discrimination at such temperatures (Huber, R., *et al.*,
Nature, **342**:833-6 (1989)).

30 Certain aspects of *M. kandleri* biochemistry place this organism aside
from other archaea. First, the membrane of *M. kandleri* consists of a terpenoid lipid
(Hafenbradl, D., *et al.*, *System Appl Microbiol*, **16**:165-9 (1993)), which is considered
to be the most primitive membrane lipid and is the direct precursor of phytanyl
diethers found in the membranes of all other archaea (Wachtershauser, G., *et al.*,
35 *Microbiol Rev*, **52**:452-84 (1988)). Second, *M. kandleri* contains a high intracellular

concentration (1.1 M) of a trivalent anion, cyclic 2,3-diphosphoglycerate, which has been reported to confer activity and stability at high temperatures to *M. kandleri* enzymes (Shima, S., *et al.*, *Arch Microbiol*, **170**:469-72 (1998)). Finally, *M. kandleri* has several unique enzymes, the most notable ones being the novel type 1B DNA
5 topoisomerase V and the two-subunit reverse gyrase (Slesarev, A. I., *et al.*, *Nature*, **364**:735-7 (1993); Belova, G. I., *et al.*, *Proc Natl Acad Sci, U S A* **98**:6015-20 (2001); Slesarev, A. I., *et al.*, *Methods Enzymol*, **334**:179-92 (2001); Kozyavkin, S. A., *et al.*, *J Biol Chem*, **269**:11081-9 (1994); and Krah, R., *et al.*, *Proc Natl Acad Sci U S A*, **93**:106-10 (1996)).

10 Perhaps the most distinctive feature of *M. kandleri* is its apparent position in the archaeal phylogeny. Several analyses, based on phylogenetic trees for 16S rRNA and the presence/absence of an 11-amino-acid insertion in EF-1 α placed *M. kandleri* close to the root of the Euryarchaeota and did not suggest any specific affinity with other archaeal methanogens (Burggraf, S., *et al.*, *System Appl*
15 *Microbiol*, **14**:346-51 (1991); Rivera, M. C., *et al.*, *Int J Syst Bacteriol*, **46**:348-51 (1996); and Nolling, J., *et al.*, *Int J Syst Bacteriol*, **46**:1170-3 (1996)). Furthermore, some signatures shared with Crenarchaeota were noticed in the 16S RNA sequence of *M. kandleri*. (Burggraf, S., *et al.*, *System Appl Microbiol*, **14**:346-51 (1991)). In contrast, the methyl coenzyme M reductase operon of *M. kandleri* consists of genes
20 that are unique to archaeal methanogens (Polushin, N., *et al.*, *Nucleosides Nucleotides Nucleic Acids*, **20**:973-6 (2001)). The genome comparison reported here reveals clustering of *M. kandleri* with the other methanogens in phylogenetic trees based on concatenated alignments of ribosomal proteins, which, together with the congruence of the sets of predicted genes, suggests that this group is monophyletic.
25 However, *M. kandleri* appears to be a "minimalist" organism whose regulatory and signaling systems are generally scaled down compared to those of other archaea. The comparative genome analysis of *M. kandleri*, *M. jannaschii* and *M. thermoautotrophicus* resulted in the delineation of a distinct set of genes characteristic of archaeal methanogens.

30 SUMMARY OF THE INVENTION

This invention provides the genomic sequences of *M. kandleri*. The sequence information is useful for a variety of diagnostic and analytical methods. The genomic sequence may be embodied in a variety of media, including computer readable forms, or as a nucleic acid comprising a selected fragment of the sequence.

35 Such fragments generally consist of an open reading frame, transcriptional or

translational control elements, or fragments derived therefrom. *M. kandleri* proteins encoded by the open reading frames are useful for diagnostic purposes, as specific and non-specific stabilizing additives for other proteins, as well as for their enzymatic or structural activity.

5 Additional objects, advantages, and novel features of this invention shall be set forth in part in the description and examples that follow, and in part will become apparent to those skilled in the art upon examination of the following or may be learned by the practice of the invention. The objects and the advantages of the invention may be realized and attained by means of the instrumentalities and in
10 combinations particularly pointed out in the appended claims.

Nucleotide or nucleic acid sequences defined herein are represented by one-letter symbols for the bases as follows:

	A (adenine)
	C (cytosine)
15	G (guanine)
	T (thymine)
	U (uracil)
	M (A or C)
	R(A or G)
20	W (A or T/U)
	S (C or G)
	Y (C or T/U)
	K (G or T/U)
	V (A or C or G; not T/JU)
25	H (A or C or T/U; not G)
	D (A or G or T/U; not C)
	B (C or G or T/U; not A)
	N (A or C or G or T/U) or (unknown)

Peptide and polypeptide sequences defined herein are represented by
30 one-letter or three symbols for amino acid residues as follows:

A/Ala (alanine); R/Arg (arginine); N/Asn (asparagine); D/Asp (aspartic acid); C/Cys (cysteine); Q/Gln (glutamine); E Glu (glutamic acid); G Gly (glycine); H/His (histidine); I/Ile (isoleucine); L/Leu (leucine); K/Lys (lysine); M/Met (methionine); F/Phe (phenylalanine); P/Pro (proline); S/Ser (serine); T/Thr

(threonine); W/Trp (tryptophan); Y/Tyr (tyrosine); V/Val (valine); X/Xaa (frame shift); and U/Sec (selenocysteine).

The present invention may be more fully understood by reference to the following detailed description of the invention, non-limiting examples of specific embodiments of the invention and the appended figures.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and form a part of the specifications, illustrate the preferred embodiments of the present invention, and together with the description serve to explain the principles of the invention.

In the Drawings:

Figure 1 illustrates the expression and purification of RPA from *E. coli* cells.

Figure 2 illustrates DNA-binding activity of RPA analyzed by 8% native PAGE, stained with fluorescein. Lane 1, RPA, 1.7 mM (I); lane 2, PDYE, 0.87 mM; lane 3, (I)+ PDYE; lane 4, (II)+ PDYE; lane 5, RPA, 2.4 mM (II); lane 6, (III)+ PDYE; lane 7, RPA, 6 mM (III).

Figure 3 illustrates Coomassie Blue G-250-stained RPA. Lane 1, RPA, 1.7 mM (I); lane 2, PDYE, 0.87 mM; lane 3, (I)+ PDYE; lane 4, (II)+ PDYE; lane 5, RPA, 2.4 mM (II); lane 6, (III)+ PDYE; lane 7, RPA, 6 mM (III).

Figure 4 illustrates the expression and purification of Ligase-1 from *E. coli* cells.

Figure 5 illustrates the expression and purification of Ligase-2 from *E. coli* cells.

Figure 6 illustrates the expression and purification of MCM2_1 from *E. coli* cells.

Figure 7 illustrates the expression and purification of Fen1 from *E. coli* cells.

Figure 8 illustrates the activity of Fen1 from MK Av19.

Figure 9 illustrates the expression and purification of Ppa from *E. coli* cells.

Figure 10 illustrates the expression and purification of RFC-S from *E. coli* cells.

Figure 11 illustrates the expression and purification of RFC-L from *E. coli* cells.

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Figure 12 illustrates the expression and purification of Pol B from *E. coli* cells.

Figure 13 illustrates DNA polymerase activity of DNA polymerase polB in various media.

Figure 14 illustrates the effect of betaine on thermostability of DNA polymerase polB in 1 M potassium glutamate at 100°C.

Figure 15 illustrates effect of potassium glutamate on the activity and processivity of DNA polymerase PolB.

Figure 16 illustrates a duplex.

Figure 17 illustrates a duplex.

Figure 18 illustrates the amplification of 110 nt region of ssDNA M13mp18(+) with ALF M13 Universal fluorescent primer (Amersham Pharmacia Biotech) and primer caggaaacagctatgacc (M13 reverse) in the presence of 1 M potassium glutamate with polB DNA polymerase.

Figure 19 illustrates the expression and purification of PCNA from *E. coli* cells.

Figure 20 illustrates the effect of PCNA on formation of fluorescent products in primer extension reaction catalyzed by polB DNA polymerase.

Figure 21 illustrates the expression and purification of Topo I from *E. coli* cells.

Figure 22 illustrates the relaxation of closed circular pBR322 DNA by Mka Topo I in 100 mM NaCl (lane 2) and 1 M KGlu (lane 5) at 80°C.

Figure 23 illustrates the expression and purification of MCM2_2 from *E. coli* cells.

Figure 24 illustrates the purification of P41P46complex from *E. coli* cells.

Figure 25 demonstrates primase activity assay for complex p41p46.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In a first aspect, the invention provides nucleic acid including the *M. kandleri* nucleotide sequence shown in SEQ ID NO. 1693 in Attachment A hereto. It also provides nucleic acid comprising sequences having sequence identity to the nucleotide sequence disclosed herein. Depending on the particular sequence, the degree of sequence identity is preferably greater than 70% (e.g., 80%, 90%, 92%,

96%, 99% or more). Sequence identity is determined as above disclosed. These homologous DNA sequences include mutants and allelic variants, encoded within the *M. kandleri* nucleotide sequence set out herein, as well as homologous DNA sequences from other *Methanopyrus* strains.

5 The invention also provides nucleic acid including sequences complementary to those described above (e.g., for antisense, for probes, or for amplification primers).

 Nucleic acid according to the invention can, of course, be prepared in many ways (e.g., by chemical synthesis, from DNA libraries, from the organism itself, etc.) and can take various forms (e.g., single-stranded, double-stranded, vectors, probes,
10 primers, etc.). The term "nucleic acid" includes DNA and RNA, and also their analogs, such as those containing modified backbones, and also peptide nucleic acid (PNA) etc.

 The invention also provides vectors including nucleotide sequences of the invention (e.g., expression vectors, sequencing vectors, cloning vectors, etc.) and
15 host cells transformed with such vectors.

 According to a further aspect, the invention provides a protein including an amino acid sequence encoded within a *M. kandleri* nucleotide sequence set out herein. It also provides proteins comprising sequences having sequence identity to those proteins. Depending on the particular sequence, the degree of sequence
20 identity is preferably greater than 50% (e.g., 60%, 70%, 80%, 90%, 95%, 99% or more). Sequence identity is determined as above disclosed. These homologous proteins include mutants and allelic variants, encoded within the *M. kandleri* nucleotide sequence set out herein.

 According to a further aspect, the invention provides highly thermostable
25 polypeptides that work in high temperature and high salt conditions where previously disclosed proteins do not.

 The proteins of the invention can, of course, be prepared by various means (e.g., recombinant expression, purification from cell culture, chemical synthesis, etc.) and in various forms (e.g., native, fusions, etc.). They are preferably prepared in
30 substantially isolated form (i.e., substantially free from other *M. kandleri* host cell proteins).

 Various tests can assess the *in vivo* immunogenicity of the proteins of the invention. For example, the proteins can be expressed recombinantly or chemically synthesized and used to screen patient sera by immunoblot. A positive reaction
35 between the protein and patient serum indicates that the patient has previously

mounted an immune response to the protein in question; i.e., the protein is an immunogen. This method can also be used to identify immunodominant proteins.

The invention also provides nucleic acid encoding a protein of the invention.

In a further aspect, the invention provides a computer, a computer memory, a
5 computer storage medium (e.g., floppy disk, fixed disk, CD-ROM, etc.), and/or a
computer database containing the nucleotide sequence of nucleic acid according to
the invention. Preferably, it contains one or more of the *M. kandleri* nucleotide
sequences set out herein.

This may be used in the analysis of the *M. kandleri* nucleotide sequences set
10 out herein. For instance, it may be used in a search to identify open reading frames
(ORFs) or coding sequences within the sequences.

In a further aspect, the invention provides a method for identifying an amino
acid sequence, comprising the step of searching for putative open reading frames or
protein-coding sequences within a *M. kandleri* nucleotide sequence set out herein.
15 Similarly, the invention provides the use of a *M. kandleri* nucleotide sequence set out
herein in a search for putative open reading frames or protein-coding sequences.

A search for an open reading frame or protein-coding sequence may
comprise the steps of searching a *M. kandleri* nucleotide sequence set out herein for
an initiation codon and searching the upstream sequence for an in-frame termination
20 codon. The intervening codons represent a putative protein-coding sequence.
Typically, all six possible reading frames of a sequence will be searched.

An amino acid sequence identified in this way can be expressed using any
suitable system to give a protein. This protein can be used to raise antibodies which
recognize epitopes within the identified amino acid sequence. These antibodies can
25 be used to screen *M. kandleri* to detect the presence of a protein comprising the
identified amino acid sequence.

Furthermore, once an ORF or protein-coding sequence is identified, the
sequence can be compared with sequence databases. Sequence analysis tools can
be found at NCBI (<http://www.ncbi.nlm.nih.gov>) e.g., the algorithms BLAST, BLAST2,
30 BLASTn, BLASTp, tBLASTn, BLASTx, & tBLASTx. See also Altschul, *et al.*,
"Gapped BLAST and PSI-BLAST: new generation of protein database search
programs," *Nucleic Acids Research*, **25**:2289-3402 (1997). Suitable databases for
comparison include the nonredundant GenBank, EMBL, DDBJ and PDB sequences,
and the nonredundant GenBank CDS translations, PDB, SwissPot, Spupdate and
35 PIR sequences. This comparison may give an indication of the function of a protein.

Hydrophobic domains in an amino acid sequence can be predicted using algorithms such as those based on the statistical studies of Esposti *et al.* Critical evaluation of the hydropathy of membrane proteins *Eur J Biochem*, **190**:207-219 (1990). Hydrophobic domains represent potential transmembrane regions or hydrophobic leader sequences, which suggest that the proteins may be secreted or be surface-located. These properties are typically representative of good immunogens.

Similarly, transmembrane domains or leader sequences can be predicted using the PSORT algorithm (<http://psort.nibb.ac.jp>), and functional domains can be predicted using the MOTIFS program (GCG Wisconsin & PROSITE).

The invention also provides nucleic acid including an open reading frame or protein-coding sequence present in a *M. kandleri* nucleotide sequence set out herein. Furthermore, the invention provides a protein including the amino acid sequence encoded by this open reading frame or protein-coding sequence.

According to a further aspect, the invention provides antibodies, which bind to these proteins. These may be polyclonal or monoclonal and may be produced by any suitable means known to those skilled in the art.

The antibodies of the invention can be used in a variety of ways, e.g., for confirmation that a protein is expressed, or to confirm where a protein is expressed. Labeled antibody (e.g., fluorescent labeling for FACS) can be incubated with intact bacteria and the presence of label on the bacterial surface confirms the location of the protein, for instance.

According to a further aspect, the invention provides compositions including protein, antibody, and/or nucleic acid according to the invention. These compositions may be suitable as vaccines, as immunogenic compositions, or as diagnostic reagents.

The invention also provides nucleic acid, protein, or antibody according to the invention for use as medicaments (e.g., as vaccines) or as diagnostic reagents.

According to a further aspect, the invention provides compositions including *M. kandleri* protein(s) and other proteins. These compositions, both covalent and non-covalent, may be more stable and may work in broader salt and pH conditions than individual proteins.

According to further aspects, the invention provides various processes.

A process for producing proteins of the invention is provided, comprising the step of culturing a host cell according to the invention under conditions, which induce

protein expression. A process which may further include chemical synthesis of proteins and/or chemical synthesis (at least in part) of nucleotides.

A process for detecting polynucleotides of the invention is provided, comprising the steps of: (a) contacting a nucleic probe according to the invention
5 with a biological sample under hybridizing conditions to form duplexes; and (b) detecting said duplexes.

A process for detecting proteins of the invention is provided, comprising the steps of: (a) contacting the antibody according to the invention with a biological sample under conditions suitable for the formation of an antibody-antigen complexes;
10 and (b) detecting said complexes.

Another aspect of the present invention provides for a process for detecting antibodies that selectably bind to antigens or polypeptides or proteins specific to any species or strain of *M. kandleri* where the process comprises the steps of: (a) contacting antigen or polypeptide or protein according to the invention with a
15 biological sample under conditions suitable for the formation of an antibody-antigen complexes; and detecting said complexes.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless
20 specified.

Directed Genomic Sequencing

A novel genome sequencing strategy was adopted to sequence *M. kandleri* strain AV19 (DSM 6324). The Sequence is listed in Attachment A as Seq ID No.: 1693.

25 *Skimming shotgun phase.* A small insert (2-4 kb) shotgun library in pUC18 cloning vector (SeqWright) was prepared from 150 µg genomic DNA of *M. kandleri* strain AV19 (DSM 6324) isolated as described (Slesarev, A. I., *et al.*, *Nucleic Acids Res*, **26**:427-30 (1998)). Approximately 1,000 purified plasmid clones and 3,000 unpurified clones (i.e., aliquots of overnight cultures) were sequenced from
30 both ends using dye-terminator chemistry (Applied Biosystems), ThermoFidelase I (Slesarev, A. I., *et al.*, *Methods Enzymol*, **334**:179-92 (2001)) and standard end Fimers (Polushin, N. *et al.*, *Nucleosides Nucleotides Nucleic Acids*, **20**:973-6 (2001); and (Polushin, N., *et al.*, *Nucleosides Nucleotides Nucleic Acids*, **20**:507-14 (2001)); (Fidelity Systems) on an ABI377. A total of 3,986 sequences, corresponding to ~0.5×
35 coverage, were assembled into 901 contigs using the Phred/Phrap/Consed software

(P. Green, unpubl., Ewing, B., *et al.*, *Genome Res*, **8**:186-94 (1998); Ewing, B., *et al.*, *Genome Res*, **8**:175-85 (1998); and Gordon, D., *et al.*, *Genome Res*, **8**:195-202 (1998)). <http://genome.washington.edu>).

5 Directed sequencing phase. The assembled contigs from the previous phase were used as islands to select Fimers for directed sequencing off the genomic DNA. Eleven rounds of Fimer selection-sequencing-assembly were performed, which allowed the genome to be assembled into 29 contigs with a 2.5× sequencing redundancy. A total of 5,499 Fimers were synthesized during this phase, from which 6,470 chromatograms were obtained. The program PrimoU
10 (<http://www.genome.ou.edu/informatics/primou.html>) was used to select priming sites at the ends of contigs.

Gap closure and assembly verification. DNA was isolated from 293 clones of the *M. kandleri* EMBL3 lambda library (Krah, R., *et al.*, *Proc Natl Acad Sci U S A*, **93**:106-10 (1996); and Slesarev, A. I., *et al.*, *Nucleic Acids Res*, **26**:427-30
15 (1998)). Remaining gaps in the genome, as well as low-quality and single-stranded regions, were closed by directed reads from genomic and lambda DNA. Fimers sequences for whole genome reads and lambda clone custom reads were selected using the Autofinish program (Gordon, D., *et al.*, *Genome Res*, **8**: 195-202 (1998); and Gordon, D., *et al.*, *Genome Res*, **11**: 614-25 (2001)). After generating 1,585
20 chromatograms, the genome was assembled into a unique contig with an estimated error rate of 0.4/10kb. This was done with 12,046 reads (~3.0× coverage). With an additional 2,147 genomic and lambda walking reads, an accuracy of less than one error per 40,000 bases was achieved (total 14,139 reads, 3.3× coverage). Lambda clones covered 85% of the genome, with an average insert size of 14,500 bp (min
25 12,230; max 19,324). There were no discrepancies between the expected insert lengths in lambda clones and the corresponding regions in the final genome sequence.

 Detailed sequencing protocols are provided for below in the Examples section.

30 **Computational genome analysis**

 The tRNA genes were identified using the tRNA-SCAN program (Fichant, G. A., *et al.*, *J Mol Biol*, **220**:659-71 (1991)) and the rRNA genes were identified using the BLASTN program (Altschul, S. F., *et al.*, *Nucleic Acids Res*, **25**:3389-402 (1997)) with archaeal rRNA as search queries. For the identification of
35 the protein-coding genes, the genome sequence was conceptually translated in 6

frames to generate potential protein products of open reading frames (ORFs) longer than 100 codons (from stop to stop). These potential protein sequences were compared to the database of Clusters of Orthologous Groups (COGs) of proteins using COGNITOR (Tatusov, R. L., *et al.*, *Science*, **278**:631-7 (1997)). After manual
 5 verification of the COG assignments and selection of start sites, the validated COG members from *M. kandleri* were considered protein-coding genes. The COG assignment procedure was repeated for ORF products greater than 60 codons obtained from the intergenic regions. Other potential protein sequences were compared to the non-redundant (NR) protein sequence database using the BLASTP
 10 program and to a six-frame translation of unfinished microbial genomes using the TBLASTN program. Those that produced hits with E (expectation) values <0.01 were added to the protein set after an examination of the alignments. Finally, protein-coding regions were predicted using the GeneMarkS (Besemer, J., *et al.*, *Nucleic Acids Res.* **29**:2607-18 (2001)) and SYNCOD (Rogozin, I. B., *et al.*, *Gene*,
 15 **226**:129-37 (1999)) programs. The genes predicted with these methods in the regions between evolutionarily conserved genes were added to produce the final protein set. (See Attachment B SEQ ID Nos.; 1-1691) 1-1688 and 1690-1692.

Protein function prediction was based primarily on the COG assignments. In addition, searches for conserved domains were performed using the
 20 CDD-search option of BLAST (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), the SMART system (<http://smart.embl-heidelberg.de/>) (Schultz, J., *et al.*, *Proc Natl Acad Sci U S A*, **95**:5857-64 (1998)) and customized position-specific score matrices for different classes of DNA-binding proteins. In-depth, iterative database searches were performed using the PSI-BLAST program (Altschul, S. F., *et al.*, *Nucleic Acids*
 25 *Res.* **25**:3389-402 (1997)). The KEGG database (<http://www.genome.ad.jp/kegg/metabolism.html>) (Kanehisa, M. *et al.*, *Nucleic Acids Res.* **28**:27-30 (2000)) was used, in addition to the COGs, for the reconstruction of metabolic pathways. Paralogous protein families were identified by single-linkage clustering of *M. kandleri* proteins after comparing the predicted protein set to itself
 30 using the BLASTP program (Makarova, K. S., *et al.*, *Microbiol Mol Biol Rev.* **65**:44-79 (2001)). Signal peptides in proteins were predicted using the SignalP (Nielsen, H., *et al.*, *Int J Neural Syst.* **8**:581-99 (1997)) program and transmembrane helices were predicted using the MEMSAT program (McGuffin, L. J., *et al.*, *Bioinformatics*, **16**:404-5 (2000)). See Table 1, Attachment C).

Gene orders in archaeal and bacterial genomes were compared using the LAMARCK program (Wolf, Y. I., *et al.*, *Genome Res*, **11**:356-72 (2001)). For phylogenetic analysis, multiple alignments of ribosomal protein sequences were constructed using the T_Coffee program (Notredame, C., *et al.*, *J Mol Biol*, **302**:205-17 (2000)) and concatenated head-to-tail. Maximum likelihood (ML) trees were generated by exhaustive search of all possible topologies using the ProtML program of the MOLPHY package, with the JTT-F model of amino acid substitutions (Adachi, J., *et al.*, *Computer Science Monographs* 27; (Institute of Statistical Mathematics, Tokyo) (1992)). Bootstrap analysis was performed for each ML tree using the Resampling of Estimated Log-Likelihoods (RELL) method (10000 replications) (Hasegawa, M., *et al.*, *J Mol Evol*, **32**:443-5 (1991)); and (Kishino, H., *et al.*, *J.Mol.Evol.*, **31**:151-160 (1990)). The likelihoods of alternative placements of *M. kandleri* in ML trees were compared using the Kishino-Hasegawa test (Kishino, H., *et al.*, *J.Mol.Evol.*, **31**:151-160 (1990)).

Design, expression, and purification of protein chimeras

The 5' to 3' exonuclease domain of Taq DNA polymerase is a structurally and functionally separate unit (Kim, Y., *et al.*, *Nature*, **274**:612-616 (1995)). Its removal produces active DNA polymerases, the Stoffel fragment and KlenTaq variants with enhanced thermostability and higher fidelity but with low processivity (Gelfand, D. H. and White, T. J. *PCR Protocols A Guide to Methods and Applications*, ed. Innis, M. A., *et al.*, (Academic Press, NY) (1990); Barnes, W. M. *Gene*, **112**:29-35 (1992)).

DNA Topoisomerase V from *M. kandleri* is an extremely thermophilic enzyme whose ability to bind DNA is preserved at very high ionic strengths (Slesarev, A. I., *et al.*, *J. Biol. Chem.*, **269**:3295-3303 (1994)). An explicit domain structure, with multiple C-terminal HhH repeats is responsible for DNA binding properties of the enzyme at high salt concentrations (Belova, G. I., *et al.*, *Proc Natl. Acad. Sci. U S A*, **98**:6015-6020 (2001); Belova, G. I., *et al.*, *J. Biol. Chem.*, **277**:4959-4965 (2002)). Thus, if the inhibition of Taq DNA polymerase, which has only one HhH motif, or its active derivatives (which lack the HhH motif) by salts is due to the inability of these enzymes to bind DNA, the transfer of HhH domain(s) derived from Topo V to Taq polymerase catalytic domain would restore the DNA polymerase at high salt concentrations.

In one embodiment, the chimeric DNA polymerase has a DNA polymerase domain that is thermophilic, e.g., is the DNA polymerase domain present

in a thermophilic DNA polymerase, such as one from the DNA polymerase in *Thermus aquaticus*, *Thermus thermophilus*, *Pfu* DNA polymerase, Vent DNA polymerase, or *Bacillus sterothrophilus* DNA polymerase. The amino acid sequence comprising one or more HhH domains, when bound to the DNA

polymerase, causes an increase in the processivity of the chimeric DNA polymerase. Five protein chimeras (also referred to herein as "hybrid proteins" "hybrid enzymes" or "chimeric constructs") containing either the Stoffel fragment of *Taq* DNA polymerase or whole size *Pfu* polymerase and a different number of HhH motifs derived from Topo V were designed. Specifically, the designed chimeras are

TopoTaq, containing HhH repeats H-L of Topo V (10 HhH motifs) linked to the N-terminus of the Stoffel fragment; TaqTopoC1 comprising Topo V's repeats B-L (21 HhH motifs) linked to the C-terminus of the Stoffel fragment, TaqTopoC2 comprising Topo V's repeats E-L (16 HhH motifs) linked to the C-terminus of the Stoffel fragment, TaqTopoC3 comprising Topo V's repeats H-L (10 HhH motifs) linked to the C-terminus of the Stoffel fragment, and *Pfu*C2 comprising repeats E-L at the C-terminus of the *Pfu* polymerase. Repeats are designated as in (Belova, G. I., *et al.*, *Proc Natl. Acad. Sci. U S A*, **98**:6015-6020 (2001). Repeats H-L (also known as Topo34) and F-L with a half of the repeat E are dispensable for the topoisomerase activity of Topo V (Belova, G. I., *et al.*, *J. Bio.. Chem.*, **277**:4959-4965 (2002) The overall structures of HhH domains are likely the same as in native Topo V, since the domains are resistant to proteolysis both in Topo V and when expressed separately (Topo 34; ((Belova, G. I., *et al.*, *J. Bio.. Chem.*, **277**:4959-4965 (2002). Also, it was thought that all Topo V domains have high internal stability in order to be functional at extremely high temperatures.

The chimeras were expressed in *E. coli* BL21 pLysS and purified using a simple two-step procedure. The purification procedure takes advantage of the extreme thermal stability of recombinant proteins that allows the lysates to be heated and about 90% of *E. coli* proteins to be removed by centrifugation. The second step involves a heparin-sepharose chromatography. Due to the high affinity of Topo V's HhH repeats to heparin Slesarev, A. I., *et al.*, *J. Biol. Chem.*, **269**:3295-3303 (1994), the chimeras elute from a heparin column around 1.25.M NaCl to give nearly homogeneous protein preparations (>95% purity). All expressed constructs possessed high DNA polymerase activity that was comparable to that of commercial *Taq* DNA polymerase.

In one embodiment, the chimeric proteins of this invention may comprise a DNA polymerase fragment linked directly end-to-end to the HhH domain. Chemical means of joining the two domains are described, e.g., in *Bioconjugate Techniques*, Hermanson, Ed., Academic Press (1996), which is incorporated herein
5 by reference. These include, for example, derivitization for the purpose of linking the moieties to each other by methods well known in the art of protein chemistry, such as the use of coupling reagents. The means of linking the two domains may also comprise a peptidyl bond formed between moieties that are separately synthesized by standard peptide synthesis chemistry or recombinant means. The chimeric
10 protein itself can also be produced using chemical methods to synthesize an amino acid sequence in whole or in part, e.g., using solid phase techniques such as the Merrifield solid phase synthesis method.

Alternatively, the DNA polymerase fragment can be linked indirectly via an intervening linker such as an amino acid or peptide linker. The linking group
15 can be a chemical crosslinking agent, including, for example, succinimidyl-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC). The linking group can also be an additional amino acid sequence. Other chemical linkers include carbohydrate linkers, lipid linkers, fatty acid linkers, polyether linkers, e.g. PEG, etc. The linker moiety may be designed or selected empirically to permit the independent interaction
20 of each component DNA-binding domain with DNA without steric interference. A linker may also be selected or designed so as to impose specific spacing and orientation on the DNA-binding domains. The linker may be derived from endogenous flanking peptide sequence of the component domains or may comprise one or more heterologous amino acids. Linkers may be designed by modeling or
25 identified by experimental trial.

As demonstrated in the discussion and examples provided below, this invention also provides methods of amplifying a nucleic acid by thermal cycling such as in a polymerase chain reaction (PCR) or in DNA sequencing. The methods include combining the nucleic acid with a chimeric DNA polymerase having a DNA
30 polymerase linked to an amino acid sequence comprising one or more helix-hairpin-helix (HhH) motifs not naturally associated with said DNA polymerase, wherein said amino acid sequence is derived from Topoisomerase V. The nucleic acid and said chimeric DNA polymerase are combined in an amplification reaction mixture under conditions that allow for amplification of the nucleic acid. Such methods are well
35 known to those skilled in the art and need not be described in further detail.

HhH domains confer DNA polymerase activity on chimeras in high salts

The polymerase activities of the four chimeras were tested by measuring initial rates of primer extension reactions. The reactions were carried out at low concentrations of substrate, when the initial rates were proportional both to total protein and PTJ concentrations. When [PTJ] is much less than Km_{app} , the initial rate is determined as in Equation 1:

$$v_1 = k_{app}/Km_{app} * [E_i][PTJ]_1 \quad \text{Eq. 1}$$

where Km_{app} and k_{app} are apparent Michaelis and catalytic constants, respectively.

The concentrations of sodium chloride (NaCl), potassium chloride (KCl) and potassium glutamate (K-Glu) were varied to assess inhibition of the Stoffel fragment and KlenTaq, and the four chimeras by salts, and to estimate the effects of the HhH domains.

Table 2 shows the inhibition constants (K_i) and the cooperativity factors (α) of *Taq* DNA polymerase, *Taq* DNA polymerase fragments (Stoffel fragment and KlenTaq), the four *Taq*-Topo V chimeras, and *Pfu* and *PfuC2* polymerases determined from the analysis of initial rates of primer extension reactions in salts using the DNA duplex of Figure 16.. Experimental values of initial polymerization rates were analyzed by nonlinear regression analysis using Equation 2:

$$v = \frac{v_o}{1 + \left(\frac{[Salt]}{K_i} \right)^\alpha} \quad \text{Eq. 2}$$

where v and v_o are initial primer extension rates with and without salt, respectively, K_i is the apparent inhibition constant; and α is the cooperativity parameter. The values for K_i and α are listed in Table 2.

In Table 2, to take into account the activation of *Pfu* polymerase and the *PfuC2* hybrid by KGlu (data entries marked with an asterisk (*)), the experimental values of initial polymerization rates were analyzed by nonlinear regression using the Equation 3:

$$v = \frac{v_o \cdot (1 + \beta \cdot [\text{Salt}]^\gamma)}{1 + \left(\frac{[\text{Salt}]}{K_i} \right)^\alpha} \quad \text{Eq. 3}$$

where v and v_o are initial primer extension rates with and without salt, respectively; K_i is an apparent inhibition constant, α is a parameter of cooperativity, β and γ are parameters of activation. Since $\gamma \cong 2$, it is likely that two ions of Glu^- bind to the *Pfu* polymerase catalytic domain without inhibiting the polymerase activity.

Table 2. Parameters of inhibition of *Taq* and *Pfu* DNA polymerases, and TopoTaq and PfuC2 chimeras by salts

Protein	NaCl		KCl		K-Glu	
	K_i	α	K_i	A	K_i	α
TopoTaq	241.3 ± 14	7.04 ± 1.4	291.1 ± 10	6.45 ± 0.6	1403.0 ± 20	6.03 ± 0.4
TaqTopoC1	228.4 ± 6	4.27 ± 0.2	231.2 ± 12	5.02 ± 0.6	1730.0 ± 125	2.45 ± 0.6
TaqTopC2	238.4 ± 3	6.77 ± 0.2	251.0 ± 6	8.97 ± 0.6	1164.5 ± 42	4.34 ± 0.5
TaqTopC3	69.0 ± 14	1.86 ± 0.2	187.7 ± 2	3.87 ± 0.1	295.8 ± 92	1.21 ± 0.2
<i>Taq</i> Polymerase	138.7 ± 6	3.24 ± 0.5	161.0 ± 6	3.50 ± 0.2	610 ± 51	4.45 ± 0.3
Stoffel Fragment	38.6 ± 3	3.45 ± 0.2	45.8 ± 4	2.92 ± 0.1	59.6 ± 38	1.47 ± 0.4
KlenTaq	40.0 ± 5	1.83 ± 0.1	32.7 ± 7	1.49 ± 0.2	71.0 ± 24	0.89 ± 0.1
<i>Pfu</i> polymerase	51.5 ± 1	2.39 ± 0.1	42.6 ± 1	3.65 ± 0.1	42.8* ± 6	3.24 ± 0.2
PfuC2	159.6 ± 33	3.62 ± 0.8	176.8 ± 3	4.68 ± 0.1	424.8* ± 9	5.76* ± 0.2

For *Taq* polymerase, inhibition constants (K_i) for NaCl and KCl are essentially the same, yet substituting KCl with KGlu increases the K_i 4-fold (Table 2). Hence, *Taq* polymerase is sensitive to anions. The cooperativity parameter α was very similar for all salts tested and suggests that as many as four anions bound simultaneously to the protein are involved.

The Stoffel and KlenTaq fragments of *Taq* DNA polymerase have almost equal sensitivities to chloride ions, which is about four times higher than the

sensitivity of *Taq* polymerase to chloride ions. Potassium glutamate inhibited these fragments only about 1.5 to 2 times less efficiently than NaCl or KCl, implying that the HhH domain can be responsible for the resistance of *Taq* polymerase to glutamate ions. It was observed that Klen*Taq* had consistently lower values of the cooperativity parameter α than the Stoffel fragment, suggesting that the additional N-terminal amino acids could mask some anion-binding sites on the catalytic domain.

As shown in Table 2, Topo*Taq* has higher inhibition constants (K_i) in salts as compared with *Taq* polymerase, and may require six to seven anions to be bound for inhibition. As a result, Topo*Taq* is active at much higher salt concentrations than *Taq* DNA polymerase. For example, a 20% inhibition of primer extension reaction occurs at about 200 mM NaCl for Topo*Taq* versus about 90 mM NaCl for *Taq* DNA polymerase. The Topo*Taq* chimera also displays little distinction between sodium and potassium cations and is less sensitive to glutamate anions versus chloride anions.

It was observed that the 21 and 16 HhH motifs at the COOH terminus of the Stoffel fragment in *Taq*TopoC1 and *Taq*TopoC2, respectively, also increase the polymerase activities of chimeras in the presence of salts. For example, 20% inhibition occurred at about 160 mM NaCl for *Taq*TopoC1 and at about 195 mM NaCl for *Taq*TopoC2. Similar to *Taq* polymerase, the *Taq*TopoC1 and *Taq*TopoC2 chimeras show no difference in inhibition by KCl versus NaCl (with the cooperativity parameter α about equal to 5), and glutamate anions were much more preferable than chloride anions. However, the cooperativity parameter for the *Taq*TopoC1 and *Taq*TopoC2 chimeras in the case of glutamate is lower compared to that of *Taq* polymerase or Topo*Taq*, suggesting that only two glutamate ions are involved in the rate inhibition.

*Taq*TopoC3 behaves differently in salts than *Taq*TopoC1 and *Taq*TopoC2. Although inhibition of *Taq*TopoC3 by KCl is similar to that of *Taq*TopoC1 or *Taq*TopoC2 (with $\alpha \approx 5$, but with a slightly lower K_i similar to that of *Taq* DNA polymerase), replacement of potassium ions by sodium ions results in a much stronger inhibition of the *Taq*TopoC3 polymerase activity and, at the same time, decreases the number of inhibiting ions to about 2. Consequently, just 30 mM NaCl inhibits the enzyme by 20%. *Taq*TopoC3 has about a fivefold relative decrease in sensitivity to K-Glu with respect to NaCl (but not to KCl), which is similar to other hybrids. However, in case of glutamate no cooperativity at all was found, suggesting that only one glutamate ion per molecule is involved in the inhibition of *Taq*TopoC3.

Introduction of C-terminal domains of Topo V into the hybrid proteins significantly extends the range of salt concentrations for the polymerase activity. This effect is due to the increase of both K_i and α , allowing chimeras to maintain their full activity at high salt concentrations. Raising the number of HhH motifs from 11 to 23 at the COOH-terminus of the Stoffel fragment made the hybrid enzymes progressively more resistant to salts. TopoTaq had the highest resistance to chloride-containing salts.

The sensitivity of *Pfu* DNA polymerase to salts was almost identical to that of Stoffel or KlenTaq fragments of DNA polymerase from *Thermus aquaticus*, possibly indicating the close functional similarity of charged amino acid residues in the active sites of these enzymes from different structural families. Attachment of Topo V HhH domains to C-terminus of *Pfu* polB significantly increased the resistance of polymerase activity to salts (Table 2). Both *Pfu* DNA polymerase and the chimera PfuC2 demonstrated virtually indistinguishable curves for KCl versus NaCl, suggesting no role for cations in inhibition. However, the Topo V domains greatly increased the resistance of *Pfu* pol activity to high levels of K₂Glu.

The invention is further illustrated by the following non-limited examples. All scientific and technical terms have the meanings as understood by one with ordinary skill in the art. The specific examples which follow illustrate the methods in which the genomic sequence, polypeptides of the present invention may be prepared and used and are not to be construed as limiting the invention in sphere or scope. The methods may be adapted to variation in order to produce compositions embraced by this invention but not specifically disclosed. Further, variations of the methods to produce the same compositions in somewhat different fashion will be evident to one skilled in the art.

EXAMPLES

The examples herein are meant to exemplify the various aspects of carrying out the invention and are not intended to limit the invention in any way.

***M. kandleri* AV19 replication factor A RPA (MK1441)**

Construction of expression vector

pET21d-M.ka-AV19-RPA: 1128 bp RPA cds was PCR-amplified from *M. kandleri* AV19 genomic DNA using following primers:

5'-ATTCCATGGGTGTGAAGCTGATGCGAACGG (SEQ ID No.:1694)

and

5'-ATAGAATTCACTCAGCTTCCTCTCCTTCACTCTCCTCC ((SEQ ID No.:1695).

NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector.

- 5 Sequencing of several inserts revealed clones carrying the correct sequence. The resulting protein sequence lacks first 56 amino acids of MK1441.

Expression and Purification of Mka RPA

- E. coli* strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 60 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38000 g for 20 minutes, heated at 75°C for 30 minutes, and centrifuged again at 38,000 g for 30 minutes. The supernatant was filtered through a 0.22 µm Millipore filter, diluted to 0.25M NaCl and applied on a Q-Sepharose column(1.6x17 cm), equilibrated with 50 mM Tris pH 7.5, containing 0.25 M NaCl and 2 mM ME. After washing with the same buffer RPA was eluted with linear gradient of 0.25-0.5 M NaCl. Fractions containing RPA were pooled, concentrated by Centriprep, followed by Centricon YM-30, and passed through a Superdex 200 (1.0x30 cm), equilibrated with 50 mM Tris-HCl pH 7.5, containing 0.15M NaCl and 2 mM ME. 15-20 mg of RPA was purified.

- Shown in Figure 1 is the expression and purification of RPA from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

DNA binding activity of RPA

- DNA-binding activity was checked with a 20-mer oligonucleotide and analyzed by native PAGE. The data is shown in Figures 21 and 22.

DNA-binding activity of RPA analyzed by 8% native PAGE, stained with fluorescein (Figure 2) and Coomassie Blue G-250 (Figure 3) RPA. Lane 1, RPA, 1.7 µM, (I); lane 2, PDYE, 0.87 µM; lane 3, (I)+ PDYE; lane 4, (II)+ PDYE; lane 5, RPA, 2.4 µM, (II); lane 6, (III)+ PDYE; lane 7, RPA, 6 µM (III).

From the experiments on titration of 1.5 μ M RPA by oligonucleotide in 1x TAE buffer pH 8.0 in the presence of 10% glycerol dissociation constant K_d was determined as described in Pavlov & Karam, 1994. $K_d = 0.21 \pm 0.15 \mu$ M.

M. kandleri strain AV19 ATP-dependent DNA ligase (MK0999)

5 Construction of an expression vector for Mka ligase (variant-1)

pET21d-Mka-AV19-Ligase1: 1896 bp DNA ligase long variant cds was PCR-amplified from *M. kandleri* (av19) genomic DNA using following primers:

5'-ATTCCATGGTAGGGGTGGTGAACGTGACTCGACCC (SEQ ID No.:1696)

10 and

5'-AATGAATTCTAGTGCTTCTGCAGTACTTCCTCGTAGATCCTCC.

(SEQ ID No.:1697)

NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several
15 inserts revealed clones carrying the correct sequence. The expressed protein contains additional Met at the N-terminus.

Expression and Purification of Mka DNA ligase (variant-1).

E. coli strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100 μ g/ml ampicillin and 34 μ g/ml
20 chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio β -galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 50 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 1mM EDTA, 5 mM β -mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at
25 38000 g for 20 minutes, filtered through a 0.22 μ m Millipore filter, diluted to 0.5 M NaCl and applied on a heparin high trap 5 ml column (APB), equilibrated with 50 mM Tris pH 8.0, containing 0.5 M NaCl and 2 mM ME. After washing the column with 50 mM Tris pH 8.0, containing 0.75 M NaCl and 2 mM ME, Ligase-1 was eluted with 1.4 M NaCl in the same buffer.

30 Shown in Figure 4 is the expression and purification of Ligase-1 from *E. coli* cells. Cell lysate before induction (lane 4), cell lysate after induction (lane 3) and purified protein (lane 2) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

Construction of an expression vector for Mka ligase (variant-2)

35 pET21d-M.ka-AV19-Lig2:

1677 bp DNA ligase long variant cds was PCR-amplified from *M.kandleri* (av19) genomic DNA using following primers:
 5'-TATCCATGGTGTACTACTCGTCCCTGGCGGAGGC (SEQ ID No.:1698)
 and 5'-AATGAATTCTAGTGCTTCTGCAGTACTTCCTCGTAGATCCTCC (SEQ
 5 ID No.:1699).

NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several inserts revealed clones carrying the correct sequence. The expressed protein contains an additional Met at the N-terminus.

10 **Expression and purification of Mka DNA ligase (variant-2).**

E. coli strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at
 15 37°C for 3 hours. The cells were harvested and dissolved in 60 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38000 g for 20 minutes, heated at 75°C for 30 minutes, and centrifuged again at 38000 g for 30 minutes. The supernatant was filtered through a 0.22 µm Millipore
 20 filter, diluted to 0.3M NaCl and applied on a heparin high trap 5 ml column (APB), equilibrated with 50 mM Tris pH 7.5, containing 0.3 M NaCl and 2 mM ME. After washing with the same buffer, the column was washed with 1M NaCl, then Ligase was eluted with 1.4 M NaCl in the same buffer. Fractions containing Ligase were passed through a Superdex 200 (1.0x30 cm), equilibrated with 50 mM Tris-HCl pH
 25 7.5, containing 0.15M NaCl and 2 mM ME.

Shown in Figure 5 is the expression and purification of Ligase-2 from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

30 **M. kandleri AV19 ATP-dependent helicase MCM2_1 (MK0965)**

Construction of an expression vector for helicase MCM2_1

pET21d-M.ka-AV19-MCM2_1:

1962 bp MCM-1 cds was PCR-amplified from *M.kandleri* (av19) genomic DNA using following primers:

5'-AATCCATGGAGCGTGAGTTCGAAGAGGCTCTCA (SEQ ID No.:1700) and
5'-AATGAATTCACATCGGGAGGTACACTCCGGGC (SEQ ID No.:1701).

NcoI-incompletely digested and EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers; additional NcoI site is presented in the cds) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several inserts revealed clones carrying the correct sequence.

Expression and purification of MCM2_1

E. coli strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 60 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38000 g for 20 minutes, heated at 75°C for 30 minutes, and centrifuged again at 38000 g for 30 minutes. The supernatant was filtered through a 0.22 µm Millipore filter, diluted to 0.3M NaCl and applied on a Q-Sepharose column(1.6x17 cm), equilibrated with 50 mM Tris pH 7.5, containing 0.3 M NaCl and 2 mM ME. After washing with the same buffer MCM2_1 was eluted with linear gradient of 0.3-1.0 M NaCl. Fractions containing MCM2_1 were pooled, concentrated by Centriprep, followed by Centricon YM-30, and passed through a Superdex 200 (1.0x30 cm), equilibrated with 50 mM Tris-HCl pH 7.5, containing 0.15M NaCl and 2 mM ME. MCM2_1-containing fractions were applied on a heparin high trap 5 ml column (APB), equilibrated with 50 mM Tris pH 7.5, containing 0.15 M NaCl and 2 mM ME. After washing column with the same buffer, MCM2_1 was eluted with linear gradient of 0.3-1.0 M NaCl in the same buffer.

Shown in Figure 6 is the expression and purification of MCM2_1 from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

M. kandleri 5'-3' exonuclease Fen1 (MK0566)

Construction of an expression vector for 5'-3' exonuclease Fen1

pET21d-M.ka-AV19-Fen1:

1077 bp Fen1 cds was PCR-amplified from *M. kandleri* (av19) genomic DNA using following primers:

5'-ATTCCATGGTTCGATCCACAGGGGTTCTGAGG (SEQ ID No.:1702)
and 5'-ATAGAATTCAGAAGAACGCGTCCAGGGTCTCTTG (SEQ ID
No.:1703).

5 NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the
primers) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several
inserts revealed clones carrying the correct sequence. The expressed protein
contains an additional Met at the N-terminus.

Expression and Purification of 5'-3' exonuclease Fen1

10 *E. coli* strain BL21 pLysS (Novagen) was transformed with expression
plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml
chloramphenicol was inoculated with transformed cells, and the protein expression
was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at
37°C for 3 hours. The cells were harvested and dissolved in 100 ml lysis buffer
containing 50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 1mM EDTA, 5 mM β-
15 mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at
38000 g for 20 minutes, heated at 75°C for 30 minutes, and centrifuged again at
38000 g for 30 minutes. The supernatant was filtered through a 0.22 µm Millipore
filter, diluted to 0.25 M NaCl and applied on heparin high trap 5 ml column (APB)
equilibrated with 0.25 M NaCl in 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM β-
20 mercaptoethanol. Fen1 was washed with the same buffer, and applied on a Q-
Sephacrose column(1.6x17 cm), equilibrated with 50 mM Tris pH 8.0, containing 0.25
M NaCl and 2 mM ME. After washing with the same buffer Fen1 was eluted with
linear gradient of 0.25-0.5 M NaCl. Fractions containing Fen1 were pooled,
concentrated by Centricon YM-30, and passed through a Superdex 200 (1.0x30 cm),
25 equilibrated with 50 mM Tris-HCl pH 7.5, containing 0.15M NaCl and 2 mM ME.

Shown in Figure 7 is the expression and purification of Fen1 from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

30 **Activity assay for Fen1.** For activity measurements of Fen1 a fluorescein - labeled oligonucleotide has been synthesized:

*FL-CTATAGGGAGACCGGAATTCGAGCTCGCCCGGGCGAGCTCGAATTCGTG
TATTTATA (SEQ ID No.:1704) which could form various secondary structures shown
below that could be cleaved by flap endonucleases:

35 Hairpins:

Most stable hairpin:
 $\Delta G = -38.11$ kcal/mol

5' CCGCTCGAGCTTAAGGCCAGAGGGATATC-FI* 5'
 5' |||||
 3' GGGCGAGCTCGAATTCCGTGTATTATA 3'

Dimers:
 10 Most stable dimer:
 $\Delta G = -85.97$ kcal/mol

5' FI*-
 15 CTATAGGGAGACCGGAATTCGAGCTCGCCCGGGCGAGCTCGAATTCCGTGTAT
 TTATA 3'
 3' |||||
 ATATTTATGTGCCTTAAGCTCGAGCGGGCCCGCTCGAGCTTAAGGCCAGAGGG
 ATATC-FI* 5'

20

Figure 8 demonstrates the activity of Fen1 from MK Av19. Lane 1 –
 Primer APAV0062 without enzymes; Lane 2 - APAV0062 after 10 minutes incubation
 with 1 u AmpliTaq in the presence of 2 mM Mg^{2+} at 55°C (positive control); Lane 3 -
 APAV0062 after 10 minutes incubation with Fen I in the presence of 1 mM Mn^{2+} at
 25 55°C.

M. kandleri AV19 Inorganic Pyrophosphatase Ppa (MK1450)

Construction of an expression vector for Inorganic Pyrophosphatase Ppa
pET21d-M.ka-AV19-Ppa:

525 bp Pyrophosphatase cds was PCR-amplified from M.kandleri
 30 (av19) genomic DNA using following primers:
 5'-TAACCATGGACCTCTGGAAAGACCTGGAACCGG (SEQ ID No.:1705) and
 5'-ATAGAATTCACCCGTGCTCCTCCTCGTACAGCT ((SEQ ID No.:1706).

NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were
 introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector.

35 Sequencing of several inserts revealed clones carrying the correct sequence.
 Expression protein starts with Met-Asp instead of Met-Asn, as it is in MK1450.

Expression and purification of inorganic pyrophosphatase Ppa

E. coli strain BL21 pLysS (Novagen) was transformed with expression
 plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml
 40 chloramphenicol was inoculated with transformed cells, and the protein expression

was induced by adding 1 mM isopropylthio - β -galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 60 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 1mM EDTA, 5 mM β -mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38000 g for 20 minutes, heated at 75°C for 30 minutes, and centrifuged again at 38000 g for 30 minutes. The supernatant was filtered through a 0.22 μ m Millipore filter, diluted to 0.25 M NaCl and applied on a Q-Sepharose column(1.6x17 cm), equilibrated with 50 mM Tris pH 8.0, containing 0.25 M NaCl and 2 mM $MgCl_2$. After washing with the same buffer Ppa was eluted with linear gradient of 0.25-1.0 M NaCl. Fractions containing Ppa were pooled, concentrated by Centriprep, followed by Centricon YM-30, and passed through a Superdex 200 (1.0x30 cm), equilibrated with 50 mM Tris-HCl pH 8.0, containing 0.15M NaCl and 2 mM $MgCl_2$.

Shown in Figure 9 is the expression and purification of Ppa from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

Ppa activity

Purified Ppa has high activity at both 20°C and 75°C using potassium pyrophosphate as a substrate in the presence of $MgCl_2$. The specific activity of the enzyme is about 250 $\mu M \cdot min^{-1} \cdot mg^{-1}$ at 20°C and 1440 $\mu M \cdot min^{-1} \cdot mg^{-1}$ at 75°C.

M. kandleri replication factor C small subunit RFC-S (MK0006)

Construction of an expression vector for RFC-S

pET21d-M.ka-AV19-RFC-S:

1905 bp RFC-S cds (containing an intein) was PCR-amplified from *M.kandleri* (av19) genomic DNA using following primers:
5'-ATACTGCAGCCATGGCCGAGCACGAGCTACGCG (SEQ ID No.:1707)
and 5'-ATAAAGCTTCTACCCGCCGGAGTACTCGTTACCGAGT (SEQ ID No.:1708).

PstI+HindIII-digested PCR fragment (PstI, NcoI and HindIII sites were introduced in the primers) was cloned into PstI, HindIII sites of pUC19 vector. A pool of isolated plasmid DNAs was used for the next round of PCR aimed to remove intein sequence. Primers

5'-GCGTTCAGCTCGAGGAAGTTGTCTCTCCA (SEQ ID No.:1709)
and
5'-CTCCGATGAGAGGGGTATCGACGTAATTCG (SEQ ID No.:1710)

were designed against the intein boundaries in the inverse orientation in order to amplify the cds region without the intein, but still containing the pUC19 sequence. The resulted PCR fragment (ca. 3.7 kb: 989 bp of cds lacking intein + 2.7 kb of pUC19 sequence) was circularized, and after transformation of *E.coli* with this vector, several plasmid DNAs were isolated and sequenced. The correct insert carrying RFC-S cds without the intein was cut out from pUC19 vector DNA by double NcoI + HindIII digestion and cloned into the NcoI+HindIII-digested pET21d vector.

Expression and Purification of RFC-S.

E. coli strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 70 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38,000 g for 20 minutes, heated at 75°C for 30 minutes, and centrifuged again at 38,000 g for 30 minutes. The supernatant was filtered through a 0.22 µm Millipore filter, diluted to 0.25M NaCl and applied on a Q-Sepharose column (1.6x17 cm), equilibrated with 50 mM Tris pH 7.5, containing 0.25M NaCl and 2mM ME. After washing with the same buffer RFC-S was eluted with linear gradient of 0.25-1.0 M.

Shown in Figure 10 is the expression and purification of RFC-S from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

M. kandleri replication factor C large subunit RFC-L (MK0006)

Construction of an expression vector for RFC-L

pET21d-M.ka-AV19-RFC-L:

1539 bp RFC-L cds was PCR-amplified from *M. kandleri* (av19) genomic DNA using following primers:

5'-AATCCATGGTAGCACCGTTGGTCCCTTGGGTTGA (SEQ ID No.:1711)
and

5'-ATAAAGCTTCAGAAGAACGCGTCTAACGTCCTCTGTTCA (SEQ ID No.:1712).

NcoI-incompletely digested and HindIII-digested PCR fragment (NcoI and HindIII sites were introduced in the primers; additional NcoI site is presented in

the cds) was cloned into NcoI, HindIII sites of pET21d vector. Sequencing of several inserts revealed clones carrying the correct sequence. The expressed protein contains an additional Met at the N-terminus.

Expression and Purification of RFC-L

5 *E. coli* strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 60 ml lysis buffer
10 containing 50 mM Tris-HCl pH 8.0, 0.6M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38000 g for 20 minutes, filtered through a 0.22 µm Millipore filter, diluted to 0.5M NaCl and applied on a heparin high trap 5 ml column (APB), equilibrated with 50 mM Tris pH 7.5, containing 0.5 M NaCl and 2 mM ME. After washing with the same
15 buffer RFC-L was eluted with shallow linear gradient of 0.5-1.0 M NaCl. Shown in Figure 11 is the expression and purification of RFC-L from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

M. kandleri AV 19 DNA polymerase family B (Mka PolB) (MK1039)

Construction of expression vector

PET21d-Mka-AV19-PolB: 2490 bp PolB cds was PCR-amplified from *M. Kandleri* AV19 genomic DNA using following primers:

5'TATCCATGGGGTTGCTCCGTACAGTGTGGGTAGATTAGCG (SEQ ID No.:1713)
25 and 5'CTAGAATTCAGCCGAAGAACTGATCCAGCGTCTT (SEQ ID No.:1714).

NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several inserts revealed clones carrying the correct sequence. The PolB protein contains a dipeptide Met-Gly at its N-terminus.

Expression and purification of Mka PolB

30 *E. coli* strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at
35 37°C for 3 hours. The cells were harvested and dissolved in 75 ml lysis buffer

containing 50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 1mM EDTA, 5 mM β -mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38,000 g for 20 minutes, filtered through a 0.22 μ m Millipore filter, diluted to 0.5M NaCl and applied on a heparin high trap 5 ml column (APB), equilibrated with 50 mM Tris pH 8.0, containing 0.5 M NaCl and 2 mM ME. After washing with the same buffer Pol B was eluted with 50 mM Tris pH 8.0, containing 0.75 M NaCl and 2 mM ME.

Shown in Figure 12 is the expression and purification of Pol B from *E. coli* cells. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

DNA polymerase activity of PolB

A primer extension assay was applied with a fluorescent duplex substrate containing a primer-template junction (PTJ). The duplex shown in Figure 18 was prepared by annealing a 5'-end labeled with fluorescein 20-nt long primer with a 40-nt long template:

DNA polymerase reaction mixtures (15-20 μ l) contained dATP, dTTP, dCTP, and dGTP (1mM each), 4.5 mM $MgCl_2$, detergents Tween 20 and Nonidet P-40 (0.2% each), fixed concentrations of PTJ – duplex, other additions, as indicated, and appropriate amounts of polB in 30 mM Tris-HCl buffer pH 8.0 (25°C). The background reaction mixtures contained all components except DNA polymerases. Primer extensions were carried out for a preset time at 75°C in PTC-150 Minicycler (MJ Research, Inc.; Waltham, MA). 5 μ l samples were removed and chilled to 4°C followed by immediate addition of 20 μ l of 20 mM EDTA. The samples were desalted by centrifugation through Sephadex G-50 spun columns, diluted, and analyzed on a ABI Prism 377 DNA sequencer (Applied BioSystems; Foster City, CA). For each sample, raw data were extracted from the sequencer trace files with the program Chromas 1.5 (Technelysium Pty Ltd., Australia), and the fluorescent signals were analyzed by our nonlinear regression data analysis programs written in Fortran. The programs applied Powell algorithms to approximate the signals by a number of Gaussian peaks and calculate integral fluorescent intensities for each product peak. The total amount of fluorescent products for each time of incubation was determined, and the initial rates of extension were calculated. PolB was found to carry out DNA synthesis at various conditions of primer extension assay.

Studies of Thermostability of pol B DNA Polymerase

To determine DNA polymerase activity and thermostability of DNA polymerase polB in various media. Proteins in 25 µl of 20 mM Tris-HCl buffer (pH 8.0 at 25°C) containing indicated concentrations of salts and betaine were incubated in PTC-150 Minicycler (MJ Research) at 95°C or 100°C. 4 µl samples were removed at defined times of incubation and assayed for primer extension activity. These activities and stabilities are shown in Figure 13.

As demonstrated in Figure 14, 1 M Betaine was found to stabilize specifically polB DNA polymerase in the presence of potassium glutamate at 100°C. The stabilizing effect of betaine is diminished in the presence of organic solvents DMSO and formamide.

It was found that potassium glutamate specifically activates polB DNA polymerase and produces about twenty-fold increase of polymerase activity at 0.8 M of the salt. See Figure 15.

Studies of processivity of Pol B DNA Polymerase

For processivity assays, the primer extension reactions were carried out and analyzed as described above, but after determination of the amount of extended products, the initial rates for appearance of each extended primer were calculated. Then the processivity for each position of the template was determined using equation:

$$P_n = \frac{\sum_{i=1}^{n_{\max}-n} v(I_{n+i})}{\sum_{i=0}^{n_{\max}-n} v(I_{n+i})}, \text{ where } v(I_{n+i}) = \frac{dI_{n+i}}{dt}, \text{ initial rate of appearance for each}$$

extended product, and the *processivity equivalence parameter*, P_e , was calculated for each reaction. Results for various concentrations of potassium glutamate are shown above.

Exonuclease activity of PolB

A 3' → 5' exonuclease activity of polB polymerase was measured at the same conditions as in the primer extension assay, except omitting dideoxynucleotides. A fluorescent primer:

^{*}FL-GTAATACGACTCACTATAGGG (SEQ ID NO.:1715)

was incubated with the enzyme at defined times. Then, the amounts of formed products were calculated, and the initial rates of hydrolysis were found, as in case of primer extension. It is interesting that polB was able to cleave off only 9 nucleotides

of the primer, that is, the 13-nt primer was the shortest substrate that polB could process.

Performance of M.K. polB DNA polymerase in various media.

Initial rates of primer extension reactions shown below in Table 3 demonstrate abolishing of 3'→5' exonuclease activity of *M.K.* polB DNA polymerase upon transformation of the enzyme into its glutamate form by buffer exchange on a Sephadex G50 column.

Table 3. Initial Rate of Primer Extension, $\mu\text{M}/\text{min}$

PolB; 0.5M NaCl	0.123±0.003
PolB; 0.5M NaCl+PCNA	0.214±0.014
PolB; 1M KGlu	2.74±0.18
PolB; 1M KGlu; dUTP	1.82±0.09
PolB; 1M DPG	2.17±0.16

The next two tables (Table 4 and 5) display effects of various media components on M.K. polB DNA polymerase activity. Initial rates of primer extension reaction were measured as described by Pavlov *et al.*, 2002.

Table 4. Initial Rate of Primer Extension, $\mu\text{M}/\text{min}$

	0.5M NaCl	1M KGlu
Pol; NaCl protein	0.15 ± 0.01	2.55 ± 0.31
Exo; NaCl protein	0.50 ± 0.06	1.07 ± 0.06
Pol; KGlu protein		2.74 ± 0.18
Exo;KGlu protein		0 ± 0

Table 5. Inhibition constants in different media

Chemical	IC ₅₀ (M)
NaCl	0.55
KCl	0.45
LiClO ₄	0.27
NH ₄ Ac	0.56
NH ₄ OH	<0.03

Conclusions:

1. KGlu inhibits the 3'-5' exonuclease activity of Mka PolB, while NaCl stimulates it.

2. KGlu , diphosphoglycerate, and Mka PCNA (see below) increase the polymerase activity of PolB.
3. PolB can use dUTP for primer extensions.
4. PolB is resistant to aggressive chemicals.

5 **Activity of Mka PolB DNA polymerase at different temperature**

Table 6. Initial Rate of Primer Extension, $\mu\text{M}/\text{min}$

$t^{\circ}\text{C}$	Initial Rates
50	1.01 ± 0.06
55	1.08 ± 0.09
60	1.12 ± 0.08
65	1.23 ± 0.05
70	1.01 ± 0.07
75	0.95 ± 0.07
80	0.92 ± 0.07
85	0.94 ± 0.07
90	0.71 ± 0.05
95	0.62 ± 0.04
100	0.62 ± 0.06
105	0.55 ± 0.09

Table 6 illustrates the dependency of initial rates of primer extension for Duplex 2 shown in Figure 17 on temperature of the reaction. Initial rates of primer extension reaction were measured as described by Pavlov *et al.*, 2002.

10 As once can see from Table 6, Mka PolB can extend primers at temperatures up to 105°C, i.e. above the melting temperature of the duplex.

Figure 18 shows the amplification of 110 nt region of ssDNA M13mp18(+) with ALF M13 Universal fluorescent primer (Amersham Pharmacia Biotech) and primer caggaaacagctatgacc (M13 reverse) in the presence of 1 M potassium glutamate with polB DNA polymerase. Cycling: 100°C for 40 seconds; 50°C for 30 seconds; 72°C for 2 minutes; 30 cycles (3, 4, 5 6). The products shown in Figure 18 were resolved on a 10% sequencing gel with ABI PRISM 377 DNA sequencer.

M. kandleri AV19 PCNA (MK1030)

20 **Construction of an expression vector for Mka DNA polymerase sliding clamp (PCNA)**

pET21a-Mka-PCNA:

PCNA was PCR-amplified from M. kandleri genomic DNA using following primers:

25 5'- ATCATTCATATGGTGGAGTTCAGGGCCTACCAG (SEQ ID No.:1716)

and

5'- AGATATGAATTCAAGGAGGAAGGGTTCACTCCT (SEQ ID
No.:1717)

NdeI+EcoRI-digested PCR fragment (NdeI and EcoRI sites were
5 introduced in the primers) was cloned into NdeI, EcoRI sites of the pET21a vector.
Sequencing of several inserts revealed clones carrying the correct sequence.

Expression and Purification of PCNA

E. coli strain BL21 pLysS (Novagen) was transformed with expression
plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml
10 chloramphenicol was inoculated with transformed cells, and the protein expression
was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at
37°C for 3 hours. The cells were harvested and dissolved in 50 ml lysis buffer
containing 50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 1mM EDTA, 5 mM β-
mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at
15 38,000 g for 20 minutes, filtered through a 0.22 µm Millipore filter, diluted to 0.25 M
NaCl and applied on a heparin high trap 5 ml column (APB), equilibrated with 50 mM
Tris pH 8.0, containing 0.25 M NaCl and 2 mM ME. PCNA was eluted with the same
buffer. Fractions containing PCNA were pooled, concentrated by Centriprep,
followed by Centricon YM-30, and passed through a Superdex 200 (1.0x30 cm),
20 equilibrated with 50 mM Tris-HCl pH 8.0, containing 0.5M NaCl and 2 mM MgCl₂.

Expression and purification of PCNA from *E. coli* cells is shown in
Figure 19. Cell lysate before induction (lane 2), cell lysate after induction (lane 3)
and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized
by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

25 Interaction of polB with PCNA.

PolB was incubated with PCNA (final concentration 5.6 µM subunits)
in the presence of 100 mM NaCl. The polymerase activity was measured in the
primer extension assay and compared to the activity without PCNA added. Even
without clamp loader, the interaction of PCNA with PolB was detected as the initial
30 rate of the primer extension increased 1.75 times. The most remarkable, however,
was suppression of hydrolysis of the primer annealed to the duplex that occurs as the
combined result of 3' → 5' exonuclease activity of polB, its sliding along PTJ, and
partial melting of the duplex substrate in the active site of the enzyme shown in
Figure 20. This happens, most likely because PCNA anchors polB on the PTJ and/or
35 prevents partial melting of the PTJ duplex.

M. kandleri AV19 DNA topoisomerase IA (Topo I) (MK1604)**Construction of an expression vector for Topo I****pET21d-M.ka-AV19-Top1:**

1761 bp Top1 cds was PCR-amplified from *M. kandleri* genomic DNA using

5 following primers:

5'-TATCCATGGCCTCGTCGTCGAAGGAGACG (SEQ ID No.:1718) and

5'-TTAGAATTCAGACCACCTTGGCTGACTTCAACTTCTTG (SEQ ID No.:1719).

NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several inserts revealed clones carrying the correct sequence.

Expression, purification, and activity of Topo I

E. coli strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 50 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38000 g for 20 minutes, filtered through a 0.22 µm Millipore filter, diluted to 0.5 M NaCl and applied on a heparin high trap 5 ml column (APB), equilibrated with 50 mM Tris pH 8.0, containing 0.5 M NaCl and 2 mM ME. After washing the column with 50 mM Tris pH 8.0, containing 0.75 M NaCl and 2 mM ME, Topo I was eluted with 1.4 M NaCl in the same buffer.

Expression and purification of Topo I from *E. coli* cells is shown in Figure 21. Cell lysate before induction (lane 2), cell lysate after induction (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

Relaxation of closed circular pBR322 DNA by Mka Topo I in 100 mM NaCl (lane 2) and 1 M KGlu (lane 5) at 80°C shown in Figure 22. Topo I was incubated with DNA for 10 min. Topoisomers were separated in a 1% agarose gel.

M. kandleri AV19 ATP-dependent helicase MCM2_2 (MK1120)**Construction of an expression vector for MCM2_2****pET21d-M.ka-AV19-MCM2_2:**

1179 bp MCM-2 cds was PCR-amplified from *M.kandleri* (av19) genomic DNA using following primers:

5'-CCATCGGTTCCGGAGGGTAGAGAGAATACG (SEQ ID No.:1720)

and

5 5'-ATTGAATTCGACTCAGGGTTTGAGCGACGAGATCCTG (SEQ ID No.:1721).

10 NcoI-incompletely digested and EcoRI-digested PCR fragment (2 NcoI sites are presented in the coding region of MCM-2 gene, from the first NcoI site the cds begins: CCATGG; the EcoRI site was introduced in the primer) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several inserts revealed clones carrying the correct sequence.

Expression of MCM2_2. *E. coli* strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio -β-galactoside (IPTG) and carried out at 15 37°C for 3 hours. The cells were harvested and dissolved in 60 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38,000 g for 20 minutes, heated at 75°C for 30 minutes, and centrifuged again at 20 38,000 g for 30 minutes.

Expression and purification of MCM2_2 from *E. coli* cells is shown in Figure 23. Cell lysate before induction (lane 2) and after induction (lane 3) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

25 ***M. kandleri* AV19 eukaryotic-type DNA primase p41p46 (MK0586 and MK1394)**

Construction of expression vectors for p41 and p46 subunits

pET21d-M.ka-AV19-p41:

948 bp p41cds was PCR-amplified from *M. kandleri* (av19) genomic DNA using following primers:

30 5'-TTACCATGGACTTCTATTCGCCAACCTTCCACAGC (SEQ ID No.:1722)

and

5'-TAAGAATTCACGGCTTAAGCTCCCCCAGCACC (SEQ ID No.:1723).

35 NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several

inserts revealed clones carrying the correct sequence. Expression protein should contain Met instead of Leu at its N-terminus.

pET21d-M.ka-AV19-p46:

1218 bp p46 short variant cds was PCR-amplified from *M. kandleri* (av19) genomic DNA using following primers:

5'-TATCCATGGGCTCATGGTTCCCCACGCCCC (SEQ ID No.:1724)

and

5'-ATAGAATTCATCCGTCGTCGGCCCTAGGTCG (SEQ ID No.:1725).

NcoI+EcoRI-digested PCR fragment (NcoI and EcoRI sites were introduced in the primers) was cloned into NcoI, EcoRI sites of pET21d vector. Sequencing of several inserts revealed clones carrying the correct sequence. Expression protein should contain Met-Gly instead of Leu-Arg at its N-terminus.

Expression of p41

E. coli strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 50 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38000 g for 20 minutes. The supernatant was filtered through a 0.22 µm Millipore filter.

Expression of p46

E. coli strain BL21 pLysS (Novagen) was transformed with expression plasmid. LB medium (2L) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with transformed cells, and the protein expression was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and carried out at 37°C for 3 hours. The cells were harvested and dissolved in 50 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.6M NaCl, 1mM EDTA, 5 mM β-mercaptoethanol, and protease inhibitors (Roche). The lysate was centrifuged at 38,000 g for 20 min, heated at 75°C for 30 minutes, and centrifuged again at 38,000 g for 30 minutes. The supernatant was filtered through a 0.22 µm Millipore filter.

Purification of p41p46 complex

p41 lysate was mixed with p46 lysate approximately 1:1 according to SDS-PAGE, heated at 80°C for 15 minutes, centrifuged at 38000 g for 15 min, and applied on Heparin- Sepharose Hi Trap 1 ml equilibrated with 50 mM Tris pH 7.5, containing 0.5 M NaCl and 2 mM ME. After washing with the same buffer

5 p41p46 complex was eluted with linear gradient of 0.5-1.0 M NaCl.

Purification of P41P46 complex from *E. coli* cells is shown in Figure 24. P41 cell lysate (lane 2), P46 cell lysate (lane 3), P41P46 complex before (lane 4) and after purification (lane 5) were analyzed by SDS-PAGE (10% gel) and visualized by Coomassie Blue G-250. Lane 1 is molecular size marker 10-225 kDa (Novagen).

10 **Assay of primase activity of p41p46.**

Primase activity assay for complex p41p46. 50 ng/μl single stranded M13 DNA (Amersham) were incubated with complex p41p46 at 75°C for 45 minutes in the presence of dNTPs (1 mM each) and MgCl₂ (4.5 mM). Then the mixture was desalted using Sephadex G-50 spin column and any primer-template junctions
15 formed by the primase were labeled with fluorescent dideoxynucleotides using SnapShot kit (ABI). The products were desalted with Sephadex G-50 spin columns and resolved on a sequencing gel using ABI 377 sequencer shown in Figure 25.

The foregoing description is considered as illustrative only of the principles of the invention. The words "comprise," "comprising," "include,"
20 "including," and "includes" when used in this specification and in the following claims are intended to specify the presence of one or more stated features, integers, components, or steps, but they do not preclude the presence or addition of one or more other features, integers, components, steps, or groups thereof. Furthermore, since a number of modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and process shown
25 described above. Accordingly, all suitable modifications and equivalents may be resorted to falling within the scope of the invention as defined by the claims which follow.

Attachment A

SEQ ID NO.1693

5 GCGTAGCGAGCGGTGTGACGTCCACTATCACCCCATCTACGACTTTCAACCTGA
CCTTTTGTCCGACCATGTGCGAAAGACGTGCACGTACACTGGGATCGACTTTGA
GTTGGAGCACTTCACCATCGCACCGCACGATGGCGGTGCCGTCTTGGATCCCC
AGAAGCACGCCCTCGATGACGGACGTCTCGTGCGGTATCTCCGGAGGTGGCG
GTACCGGTGAGGCGACCGCCGCCAGGATTAGGACGGGGGTAAACCCGACCGG
10 CAACCGGACCGGCCCTCATCGAGCGGCCACCTCTCGGCTCGTCGTAGTAT
CATCTTGGTGGTGAGGCCGGATACGGACTCAGGTCGGAGTACTTCTCGAGCCC
GATAAGCGGAGGGGGGTGCGGACGCCAAGATTACTATCGAGTGAAAGATCCGC
GGATGCTTCCTCACTGCATCGATAAGCATGGACCTCCAAAATTTTTCCCTACCCA
GGGAGAACCAGTCGTCTGGATGATCCCGAGGGTCCCTAGGTCGTGAGCTGTT
15 AACTCTTCTCCGAGAGGGAGTAACTCGCGGGCTAACTCCTCACCCCGTTGGTG
GACCCGCTTAGCCACTGACGCGGGCACGCGGGCTTCGGACCGAATACGTGTC
CAGTGTCTTCCGAAACAGTTCACGCCGAAGCGCAGAAAAATCTGTTTGGAGTAG
TGGGGAGCCGTTTTGCGCAGTTCGCGCTACACGTCTACGTCGGATGTGAACAT
CGCTCAACCCTCGGGCCGAACCTTCGACTGTGCGGGGCTCCCCCTCTTCCCGC
20 GGATTTGACGCTCACGGGGAGGAATTTCTCCACCACCCACGCGTTTCGTGGG
AGGTGCGGATCGACACCGGTTACACCGAAGACCGATTACCGTCGGCGATCGC
TAGGAATGGCAGGATCTGATCTCCCATGTGCTCGTCAAGGGCCATTCCAGTGC
GAAGACGCTGCACGAGCTGCTCCGCGGCCTCTCGTCCACACCTCCGCGGG
CTTGCCCTTCTCACCCAGCGCATCGGCACCGATCCTGTTCCCTTGATCGTCTTC
25 CGCCCATAGACGATACCGCTGCCGGGACCTAGGTGTGGATCGCGGCCTTTAG
GGTACGTCTCGATCTCGATTTAGGTCTGATCCCGAGCTCCCTCTCGATAATTT
CCGAAGCTGCCTTGGCCTGACGCTCCGCCACGTGGGGTGGTAACCGCACGCA
GTGGGAGATCCCTCGAACGCTCTCCAGCTCGCCGAACCTTCACGGCTTCCAGCG
GCTTCAGCCGCTTCGGGGGTTCCATCCTGGCCCTGACTATCCCTCCACCCCGA
30 GGATAATGGCCGCGTCGCAGGACCTCGAGCTCGTACCGGTAACCGAGGCGGT
CTAGGTAGTGTGCGTTGACGTTGATCTCGTAGTCCACGGGCGGTGACCACTTG
ACGTCCGTCCCGCCTCGCACTTCCATCTCCACGGGCCCGTCGGCGGCTATCGC
CGCGAGCTTGACGGCTTGAAGCAGTAGGGTCACGCTACCCGCGGTACCGATAT
CTACCTCGTACTCCCTCCTTTACCTTCCCCGGCTCGAACACGATCTCGGTTCG
35 AACCTATCTCGAGTCCCTCGCACTCCGCATCGCAGATCTCGGCCACGGCCTTC
ACTGCGTGTAGGTGCTGGTGTGAGAGGCCGGGACGTGGCCTGTTGGCCCGGA
TGTGTATATCCGGACGGGTTACCTGTGACGCTGACATCCCGACCGCCGTT
CGGAGTATCTGGCCTCCACCCTACCGTAGGAACCGTCGATCTCTATCAAGCTC
ATCACCGGCACCGGCGATGAGGAGGGTCCGGATGGCCGCGCTCGTGAGGAGA
40 GAACCCGGGGCTGAGCCGATCTTAACGCCGTCACTCTTCCTCCATCGGTTTC
ACTTCTATTTCACTGGCTTCCGCCTTCGGCGCCCTCACGATCAGGAGACCACGA
CCGCACTTCGCACTGACGGCGCTCGGGTTGATTTTCTCCGGTAAGTCTATCCTC
CGTCGGACCTCGCCGGTCACGCGCTCCCGCGTCTTGGCCTCACCTCTCGCAT
CTTCGGGATGTTAGCCGTGATCTCCACGAACCTCTCACCCGCTCTGACCTGAAC
45 GTCCTCCGGGCGGGCGCCGGGAACCTCCGCGACGATGACGAACCTCTCCCGAA
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